NOTE TO THE FILE
BNF 0036
December 11, 1998

Subject: Male sterile corn lines 676, 678, and 680

Keywords: DNA adenine methylase (DAM), *E. coli*, anther-specific promoter, phosphinothricin acetyltransferase (PAT), *Streptomyces viridochromogene*, male sterility.

Background

On June 20, 1996, Pioneer Hi-Bred International, Inc. (Pioneer) initiated a consultation with FDA regarding its development of male sterile corn lines. In submissions dated April 15, 1998, June 18, 1998, and November 16, 1998, Pioneer provided summary information to support its safety and nutritional assessment of its new transgenic corn lines 676, 678, and 680, derived from initial transformation events TC676, TC678, and TC680.

Intended Effect and Food/Feed Use

According to Pioneer, hybrid maize varieties have a number of advantages over non-hybrid varieties, such as increased vegetative growth and increased yield. Traditionally, the pollen-bearing tassel is removed from the inbred designated as the female (detasseled) before anthesis (pollen shed) so that the inbred female will only be fertilized with pollen from the inbred male. One row of the male inbred is planted for every four to six rows of the female inbred. Therefore, the resulting seed is hybrid and will form hybrid plants. An alternative approach has been the development of "male sterile" plants through the use of naturally occurring cytoplasmic male sterility (CMS). However, the CMS trait in the hybrid plants is sometimes associated with reduced agronomic performance. To produce corn lines with controlled fertility, Pioneer developed corn lines that lack pollen and are phenotypically male sterile by using a Tissue Specific Sterility (TSS) system. TSS corn lines contain the gene designated *dam* which encodes the DNA adenine methylase (DAM methylase) from *Escherichia coli*. The gene appears to be expressed only in the anther cells of the maize, due to the use of the corn 5126del promoter, and disrupts normal cell function, including the ability to produce anthers and pollen. Plants containing *dam* are utilized as the female parent for hybrid seed production, as they are unable to produce pollen.

The *dam* gene is linked to the selectable marker gene, *pat*, which confers tolerance to the herbicide, glufosinate ammonium. The *pat* gene, derived from *Streptomyces viridochromogenes*, encodes the enzyme phosphinothricin acetyltransferase (PAT) which acetylates phosphinothricin (the active ingredient in the herbicide glufosinate), and thereby confers tolerance to the herbicide. Since *dam* and *pat* are physically linked, these genes segregate as a single locus. Because of this linkage, judicious use of glufosinate to emerging maize plants allows only male sterile plants to grow in the seed increase fields. Crossing male sterile (DAM) maize with other inbred lines results in a population that is only 50% male sterile and thus, the nonsterile plants

must be removed prior to pollination. Consequently, the male sterile line can be maintained by cross-pollinating with wild-type plants followed by an application of glufosinate. The linked insertion confines use of the herbicide to breeding and selection purposes. The hybridization technique used for the production of commercial hybrids is based on the male sterility of one of the female parental lines. In the hybrid, the new traits (male sterility and tolerance to glufosinate) are expected to be present in 50% of the plants.

Corn is one of the world's primary cereal grain crops. Corn grain (kernels) has both animal feed and human food applications. Human food uses of the grain include: 1) direct consumption of the kernels, which constitutes 2-3% of total domestic corn consumption; and 2) the productions of high fructose corn syrup, glucose, corn oil, starch, ethanol and corn meal, which constitute about 20% of total domestic corn consumption. More than 75% of domestically produced corn grain is used in animal feed, primarily for poultry, swine, and cattle. Animal feed uses for corn include: 1) the grain, fed whole or in a processed form; 2) the byproducts from the production of human food; and 3) silage which is produced from the entire corn plant.

Molecular Alterations and Characterization

Pioneer used microprojectile bombardment to introduce one plasmid into hybrid corn tissue. The plasmid, PHP6710, is based on the pUC18 plasmid and contains three genes: 1) the DNA adenine methylase (*dam*) gene, which is the basis of the tissue specific sterility (TSS) system for male sterility; 2) the phosphinothricin acetyltransferase gene (*pat*) for use as a selectable marker; 3) and the ampicillin resistance gene (*bla*) which expresses β-lactamase for selection of the plasmid in bacteria.

The *dam* gene is under the control of the maize promoter 512del, which is an anther-specific promoter, and the *Solanum tuberosum* proteinase inhibitor II transcription terminator (pinII). The *pat* gene is under control of the cauliflower mosaic virus 35S promoter (CaMV 35S), which stimulates high expression of genes under its control.

Prior to micro-projectile bombardment, Pioneer digested the plasmid with the restriction enzyme *RcaI* to isolate a 4.5 kb DNA fragment containing the coding regions for *dam* and *pat*, but not *bla*, and used this DNA fragment for transformation of corn tissue. Southern analyses confirmed the absence of the *bla* gene from the DNA preparation used to transform as well as from the genomic DNA of the transgenic corn.

Pioneer performed Southern blot analyses to determine the presence and copy number of genes introduced into the genome of the transgenic corn lines. All three lines, 676, 678, and 680, contain one complete copy of *dam* and *pat* at one insertion site. Line 676 contains a second *pat* at another insertion site within the corn genome. Line 678 contains two additional copies of *dam* and one additional copy of *pat*. According to Pioneer, the second *pat* appears to be a partial copy and one *dam* appears to have been rearranged, leading to loss of an internal restriction enzyme site. Line 680 contains three partial copies of *dam* in addition to the full length copy. The *pat* gene in this line was modified outside of the coding region, but within the terminator sequences.

Expressed Proteins

Pioneer used northern blot analyses to examine tissues for the presence of mRNA in the transgenic corn lines. Testing the tassel of corn lines 676 and 680 showed no dam-specific mRNA. Pioneer considered its inability to detect dam-specific mRNA to be consistent with the use of a restrictive promoter for dam expression and the fact that DAM methylase results in cell ablation and destruction of anther tissue. However, tissue from the tetrad release stage of male sterile corn line 678 contained detectable dam-specific mRNA. Pioneer did not anticipate expression of the *dam* gene in other tissues. Northern blot analysis confirmed that no dam-specific mRNA was detected in leaf tissue from corn lines 676, 678, and 680.

To confirm that the dam-specific mRNA is produced in the anther but not detectable due to cell ablation and destruction, Pioneer tested for dam-specific mRNA in three corn lines with a frame shift mutation in the *dam* gene. The frame shift mutation allows expression of a truncated DAM methylase that is nonfunctional and does not result in cell ablation. Northern analyses of tassel tissue from these plants showed the presence of dam-specific mRNA. According to Pioneer, these results are consistent with the phenotypic evidence that *dam*, under the control of an anther-specific promoter, functions in maize in a tissue specific manner during the tetrad release/early vacuolate stages of pollen development, and thereby, prevents the production of pollen in male sterile corn lines 676, 678, and 680.

Pioneer also analyzed PAT protein expression in grain and leaf tissue of male sterile corn lines 676, 678 and 680. Leaf tissue from lines 676 and 678 contained 601-717 and 204-278 μg per gram of total protein, respectively. The amount of PAT in leaf tissue from line 680 was below the detection limit of 20 μg per gram of total protein although it is sufficient to confer tolerance to glufosinate. PAT was not detectable in grain from line 680. In grain from the other lines, the amount of PAT per gram of total protein ranged from being non-detectable to 19.2 μg for line 676, and 14.8 μg in line 678. Pioneer concludes that PAT is present in very low amounts in grain from male sterile corn lines 676, 678, and 680.

Safety of the Introduced Proteins

The tissue specific promoter for *dam* ensures that expression occurs only in anthers, not in grain, forage, or other plant tissues. The observed cell ablation in the anther tissues of male sterile corn lines ensures that the presence of DAM methylase is limited to the anthesis stage of plant development. Therefore, Pioneer indicates that the levels of DAM methylase in food and feed are expected to be minimal. Pioneer states that even if it were to be present in food or feed, there is no reason to expect that DAM methylase is toxic to humans or animals. Methylase enzymes are found in both prokaryotes and eukaryotes and are widely consumed by humans and animals in foods and feeds. In addition, Pioneer found no significant homology between the DAM methylase sequence and known toxins or allergens in the GenBank, PIR and SwissProt sequence databases.

Pioneer notes that use of the *pat* gene and protein in corn have been the subject of previous food and feed safety assessments in other completed consultations with FDA. Those safety assessments concluded that there was no concern regarding the safety of PAT in glufosinate-tolerant corn lines. Pioneer also notes that the level of PAT in male sterile corn lines 676, 678, and 680 does not exceed those levels observed in other commercially available glufosinate-tolerant corn lines. Based on acute oral toxicity studies, and *in vitro* digestibility studies, Pioneer states that PAT does not pose any significant human health risk.

Compositional Analysis

To test the nutritional content of the grain and forage from male sterile corn lines 676, 678, and 680, Pioneer produced an F1 generation of hybrid corn plants in which the female parent was from the male sterile corn lines. To be representative of the transgenic lines, Pioneer used the hybrid corn plants that contained the male sterility trait. As isogenic, non-transgenic control lines, Pioneer used the hybrid plants that were male fertile.

For grain and forage, Pioneer performed analyses for the content of protein, fat, ash, calcium and phosphorus. Pioneer also analyzed for forage crude fiber. Pioneer reports that its method for chopping and grinding forage samples included a drying step which removes most of the moisture from the forage. In forage, Pioneer reports that the levels of the analyzed components were comparable between the male sterile lines and the control as well as values reported in the published literature. Pioneer measured forage crude fiber levels, but not the amounts of acid detergent fiber (ADF) and neutral detergent fiber (NDF). However, Pioneer states that, based on data obtained from 262 samples of non-transgenic hybrids, crude fiber levels correlate well with amounts of ADF, but not NDF. While the firm does not anticipate any unusual findings, Pioneer intends to measure ADF and NDF levels in forage from the three transgenic corn lines to confirm that there is no difference between the transgenic corn plants and their non-transgenic counterparts in these values.

For grain, Pioneer found that levels of the analyzed components were comparable between the male sterile lines and the control. Except for calcium, levels of the analyzed components were also in the range of values reported in the published literature. Calcium levels were comparable between the male sterile line and the control, but lower than values reported for corn grain in the published literature. Pioneer notes that this difference is likely due to the analytical methodology used to measure calcium by its contract laboratory. The method calls for ashing whole grain rather than preground grain. While this method eliminates sample-to-sample contamination, it gives lower calcium values compared to ashing of preground grain.

Pioneer conducted an analysis of the five major fatty acids in corn, namely, palmitic (C16:0), stearic (C18:0), oleic (C18:1), linolenic (C18:2), and linolenic (C18:3). Pioneer reports that the levels of these fatty acids in the three transgenic corn lines were all within the range of values in the published literature, indicating that the new corn lines are comparable to commercial corn lines in fatty acid composition.

Pioneer also analyzed the levels of seventeen amino acids in grain from the three corn lines and states that, in general, the levels were within the range of values in the published literature, indicating that these lines are comparable to commercial corn hybrids with respect to amino acid composition. Pioneer attributes the few instances where the value of an amino acid was slightly lower or higher than published values to the genetic background of the inbred parental lines.

Conclusions

Pioneer has concluded that the corn lines containing transformation events TC676, TC678, and TC680 are not materially different in terms of food safety and nutritional profile from corn varieties currently on the market. At this time, based on Pioneer's description of its data and analyses, the Agency considers Pioneer's consultation on new corn lines, 676, 678, and 680, to be complete.

Nega Beru, Ph.D.